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Plasmid-Associated Virulence of *Salmonella typhimurium*

PAUL A. GULIG AND ROY CURTISS III*

Department of Biology, Washington University, St. Louis, Missouri 63130

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We investigated the role of the 100-kilobase (kb) plasmid of *Salmonella typhimurium* in the virulence of this organism for mice. Three strains, LT2-Z, SR-11, and SL1344, which possessed 100-kb plasmids with identical restriction enzyme digestion profiles, were cured of their respective 100-kb plasmids after Tn*mini-tet* was used to label plasmids. Curing wild-type virulent strains SR-11 and SL1344 raised peroral 50% lethal doses from 3 × 10^5 and 6 × 10^4 CFU, respectively, to greater than 10^8 CFU. Both wild-type strains had intraperitoneal 50% lethal doses of less than 50 CFU, whereas the intraperitoneal 50% lethal doses for cured SR-11 and SL1344 were less than 50 and 400 CFU, respectively. Reintroduction of the Tn*mini-tet*-labeled, 100-kb plasmid restored wild-type virulence. Invasion from Peyer's patches to mesenteric lymph nodes and spleens after peroral inoculation was the stage of pathogenesis most affected by curing *S. typhimurium* of the 100-kb plasmid. Wild-type *S. typhimurium* replicated in spleens of mice inoculated intravenously to a greater extent than did plasmid-cured derivatives. Wild-type and cured strains equally adhered to and invaded Henle-407, HEp-2, and CHO cells; furthermore, the presence of the 100-kb plasmid was not necessary for replication of *S. typhimurium* within CHO cells. The 100-kb plasmid had no effect on phagocytosis and killing of *S. typhimurium* by murine peritoneal macrophages in vitro and in vivo. Similarly, wild-type and plasmid-cured strains were resistant to killing by 90% normal human, rabbit, and guinea pig sera. All wild-type and plasmid-cured *S. typhimurium* strains possessed complete lipopolysaccharide, as determined by silver staining solubilized cells in sodium dodecyl sulfate-polyacrylamide gels. We have confirmed the role of the 100-kb plasmid of *S. typhimurium* in virulence, primarily in invasion to mesenteric lymph nodes and spleens after peroral inoculation of mice. Involvement of the 100-kb plasmid in infection of mesenteric lymph nodes and spleens suggests a role for the plasmid in the complex interaction of *S. typhimurium* with cells of the reticuloendothelial system.

Salmonella typhimurium is the leading cause of human disease among *Salmonella* serotypes (10). The *S. typhimurium* mouse model for human typhoid fever caused by *S. typhi* was described by Carter and Collins (9) and others (11, 12, 29, 60). *S. typhimurium* colonizes the Peyer's patches of the small intestine and invades to draining mesenteric lymph nodes, where *S. typhimurium* is resistant to killing by phagocytic cells (5, 11) and spreads through the lymphatic system to the spleen and liver. Because *S. typhimurium* is resistant to the bactericidal activity of serum complement (37, 38), bacteremia and death ensue. Dowman and Meynell (19) and others (1, 3, 53, 54, 57, 59) reported the presence of a cryptic plasmid in some *S. typhimurium* strains. This plasmid encoded fertility inhibition (57) and could integrate into the chromosome to drive chromosomal replication in *dnaA* mutants (3). Jones et al. (33) found that a plasmid of approximately 60 megadaltons (100 kilobases [kb]) encoded virulence traits in *S. typhimurium* strains of clinical origin. They determined that the plasmid was involved in mannose-resistant adherence to and invasion of HeLa cells. Subsequently, Helmuth et al. (27) determined that presence of high-molecular-weight plasmids in various *Salmonella* serotypes correlated with resistance to bactericidal activity of normal human serum. Hackett et al. (22) confirmed the role of the cryptic plasmid in serum resistance of *S. typhimurium* but found no correlation between the presence of the plasmid with adherence to tissue culture cells or colonization of Peyer's patches after peroral (p.o.) inoculation. Pardon et al. (45) determined that the *S. typhimurium* virulence plasmid was involved in invasiveness of *S. typhimurium* from mesenteric lymph nodes to spleens after p.o. inoculation of mice. Peyer's patch colonization and mesenteric lymph node infection were equal between wild-type and plasmid-cured strains of *S. typhimurium*. Recently, Hackett et al. cloned a gene from the virulence plasmid that conferred resistance to normal human serum to both plasmid-cured *S. typhimurium* and *Escherichia coli* K-12 (23).

In this report, we confirm the relationship of the 100-kb plasmid to virulence of *S. typhimurium*. In contrast to others (22, 23, 27, 33), we found no role of the 100-kb plasmid in serum resistance or mannose-resistant adherence of *S. typhimurium* to tissue culture cells. We confirmed the results of others that the 100-kb plasmid was not necessary for colonization of Peyer's patches but was involved in infection of spleens (22, 45) and mesenteric lymph nodes after p.o. inoculation of mice. These data suggest a role for the virulence plasmid in interactions between *S. typhimurium* and macrophages; however, in vitro and in vivo infection of murine macrophages failed to demonstrate a difference in phagocytosis or killing between wild-type and plasmid-cured *S. typhimurium*.

(A preliminary report of these data was presented at the Interscience Conference on Antimicrobial Agents and Chemotherapy in 1986 [P. A. Gulig and R. Curtiss III, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1171, 1986]).

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1 along with plasmid descriptions. Three lines of *S. typhimurium* were used: mouse-passaged, virulent strains SR-11 (52) and SL1344 (30) and the less virulent strain LT2-Z (65). *E. coli* HB101 (7) and K-12 derivative χ2934 were used in genetic manipulations.

* Corresponding author.

TABLE 1. Bacterial strains

Strain	100-kb plasmid	Genotype	Description
S. typhimurium			
LT2-Z			
χ3000	pStLT100	Wild type	(65) Obtained from C. Turnbough
χ3147	pStLT100	*gyrA1816*	Spontaneous Nal^r derivative of χ3000
χ3344			100 kb plasmid-cured χ3000
χ3347	pStLT101		pStLT101 (Tn*mini-tet*-labeled pStLT100 obtained by transposition from pNK861) transformed into χ3344, Tet^r
χ3477		*hsdL6* Δ(*galE-uvrB*)-*1005 flaA66 rpsL120 xyl-404 lamB*$^+$ (*E. coli*) Δ(*zja*::Tn*10*) *hsdSA29*	Used to obtain Rc chemotype LPS; derived from AS68 of T. Palva by S. A. Tinge and R. Curtiss (unpublished); Δ(*galE-uvrB*)-*1005* obtained from B. A. D. Stocker in P22 HT*int* lysate from SL54000
SR-11			
χ3181	pStSR100		SR-11 (52) isolated from Peyer's patch of an infected mouse; from Suzanne Michalek
χ3306	pStSR100	*gyrA1816*	*gyrA1816* transduced into χ3181 from χ3147; mouse passaged
χ3337		*gyrA1816*	100-kb plasmid-cured χ3306
χ3338	pStSR101	*gyrA1816*	pStSR101 [Tn*mini-tet*-labeled pStSR100 obtained by transduction from χ3000(pStLT101)] transformed into χ3337, Tet^r
χ3456	pStSR101		χ3181 with Tn*mini-tet*-labeled pStSR100 obtained by transduction from χ3000(pStLT101), Tet^r
SL1344			
χ3042	pStSL100	*rpsL hisG*	From B. A. D. Stocker (30)
χ3339	pStSL100	*rpsL hisG*	Mouse-passaged χ3042
χ3340		*rpsL hisG*	100-kb plasmid-cured χ3339
χ3351	pStSR101	*rpsL hisG*	pStSR101 (Tn*mini-tet*-labeled pStSR100) transformed into χ3340, Tet^r
E. coli			
K-12 χ2934		F$^-$ *thr-1 leuB6 tonA1 lacY1* λ$^-$ *gyrA thi-1*	Spontaneous Nal^r derivative of C600 (2) H. A. Lockman and R. Curtiss (unpublished data)
HB101 χ2642		F$^-$ *ara-14 leuB6 proA2 lacY1 glnV44 galK2* λ$^-$ *recA13 rpsL20 xyl-5 mtl-1 thi-1 hsdS20* (r_B^- m_B^-)	(7)

Culture media and growth conditions. Unless stated otherwise, bacteria were grown in L broth or on L agar plates (36) supplemented with appropriate antibiotics at the following concentrations (micrograms per milliliter): ampicillin, 100 to 200; tetracycline, 12.5 to 25; chloramphenicol, 30; kanamycin, 50; streptomycin, 50; and nalidixic acid, 50. For some adherence, invasion, and macrophage infection assays some cultures were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.), because this medium increased bacterial adherence to the mammalian cells used. All bacteria were grown overnight at 37°C in static broth cultures of the appropriate medium and were subcultured into shaking broth cultures until the late-logarithmic phase of growth (optical density at 600 nm, approximately 0.4 to 0.7).

Genetic exchange. Transformation was performed by the method of Humphreys et al. (31). Phage P22 HT *int*-mediated transduction was performed as described previously (51). Conjugations were performed either by plate matings or filter matings (63).

DNA manipulations. Large-scale and rapid minilysate plasmid extractions were performed by the method of Birnboim (4). Cesium chloride density gradient centrifugation, Southern blot hybridization, colony blot hybridization, and agarose gel electrophoresis were performed by standard procedures (42). Nick translation of DNA with [α-^{32}P]ATP (Amersham Corp., Arlington Heights, Ill.; specific activity, 1,445 Ci/mmol) was with the Bethesda Research Laboratories (Gaithersburg, Md.) kit according to the manufacturer's instructions. Restriction enzyme digestions were with enzymes from Bethesda Research Laboratories or International Biotechnologies, Inc. (New Haven, Conn.) according to the manufacturer's instructions.

Labeling the *S. typhimurium* 100-kb plasmid with Tn*mini-tet*. Strain χ3000, which contained plasmid pStLT100, was transformed with pNK861, which possesses Tn*mini-tet* (62). Tn*mini-tet* is essentially the Tet^r gene of Tn*10* within inverted repeats. pNK861 was excluded from the library of Amp^r Tet^r χ3000(pStLT100, pNK861) by mobilizing in the incompatable plasmid pNK259 from χ3000(pNK259, F::Tn*5*) with F::Tn*5*. To select for Tn*mini-tet* insertions into the 100-kb plasmid, which is mobilizable by F (33), the χ3000::Tn*mini-tet* (pNK259, F::Tn*5*) library was mated with *E. coli* HB101 selecting for Tet^r, Kan^r, and Str^r. Potential Tn*mini-tet*-labeled, 100-kb plasmids in HB101 were mobilized back into *S. typhimurium* χ3147 by selecting for Tet^r and Nal^r. Several Nal^r Tet^r Kan^s isolates which did not possess F::Tn*5* were picked.

Tn*mini-tet* insertions in the 100-kb plasmid were transduced into each of the 100-kb plasmid-containing, wild-type *S. typhimurium* strains by phage P22 HT *int*-mediated transduction. Tet^r transductants were screened for increased size of the 100-kb plasmid and alteration in the restriction enzyme profile from that of the parental plasmid.

An alternative method for isolating pStLT100::Tn*mini-tet*

insertions was to obtain plasmid DNA from χ3000(pStLT100, pNK861) and to transform *E. coli* HB101(pNK259), selecting for Tet^r and Cam^r.

Curing *S. typhimurium* of the Tn*mini-tet*-labeled, 100-kb plasmid. *S. typhimurium* strains with Tn*mini-tet* insertions in the 100-kb plasmid were subjected to two curing regimens—growth at 43°C or growth in L broth containing novobiocin (200 to 250 μg/ml). Tn*mini-tet*-labeled strains were passaged daily with low inocula (10^3 to 10^4 CFU) for each of the curing regimens. When cultures had reached the stationary phase (optical density at 600 nm, approximately 0.9), a portion was diluted and plated on medium containing fusaric acid (6, 41). Fusaric acid-resistant colonies were screened for Tet^s, and the plasmid contents of fusaric acid-resistant, Tet^s colonies were examined by minilysate analysis (4). Cured derivatives were further examined by Southern blot hybridization of minilysates and colony blot hybridization of bacterial cells with ^{32}P-labeled pStSR100 to confirm curing and lack of chromosomally integrated plasmid.

Mouse infections. Female BALB/c mice 7 to 10 weeks old (Harlan Sprague Dawley, Indianapolis, Ind., and Sasco Inc., St. Louis, Mo.) were used for all animal infections. Older mice were used to obtain peritoneal macrophages. Normal mouse serum was obtained from male mice of various ages. Mice were housed in filter-topped cages with raised wire floors to prevent cross-contamination.

For p.o. inoculation, mice were starved for food and water for 6 h and then fed 50 μl of 10% (wt/vol) sodium bicarbonate, followed by 20 μl of bacteria suspended in buffered saline containing 0.1% (wt/vol) gelatin (BSG) (15). p.o. inoculation was with a micropipette tip placed directly behind the incisors to avoid damage to the oral mucosa. Mice were fed food and water 30 min postinoculation.

For intraperitoneal (i.p.) inoculation and lateral tail vein intravenous (i.v.) inoculation, mice were injected with 0.1 to 0.2 ml of bacteria suspended in BSG.

Organs and tissues of infected mice were examined for presence of *S. typhimurium* as follows. Mice were killed by CO_2 asphyxiation. Spleens were aseptically removed and homogenized in 2.5 ml of BSG in either an Omni-Mixer equipped with a microcup (Du Pont Co., Wilmington, Del.) or a glass tissue homogenizer at room temperature. Peyer's patches were removed from small intestines and washed twice by vortexing in 2 ml of BSG. Rinsed Peyer's patches were homogenized in 2.5 ml of BSG either in an Omni-Mixer or by vortexing in glass tubes containing glass beads. Mesenteric lymph nodes were homogenized in 2.5 ml of BSG by vortexing with glass beads. Dilutions of tissue and organ homogenates were plated on L agar plates containing appropriate antibiotics.

Mouse 50% lethal dose (LD_{50}) values were determined by the method of Reed and Muench (47) with four to six mice per inoculum dose.

Henle-407, HEp-2, and CHO cell adherence and invasion. Henle-407, HEp-2, and CHO cells (25) were grown in 24-well culture plates with Eagle minimal essential medium (Sigma Chemical Co., St. Louis, Mo.) supplemented with 10% (vol/vol) fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) containing penicillin (100 U/ml) and streptomycin (100 μg/ml) (Sigma). Monolayers were grown to confluency, approximately 5×10^5 cells per well, as determined by counting trypsin-released cells in a hemocytometer. Monolayers were rinsed with Eagle minimal essential medium without antibiotics and incubated with Eagle minimal essential medium without antibiotics for at least 2 h before infection with *S. typhimurium*. Monolayers were rinsed with Hanks balanced salt solution (Sigma) immediately before infection. Bacteria were centrifuged at 12,000 × *g*, suspended in BSG, and diluted into Hanks balanced salt solution with or without 1% (wt/vol) D-mannose or 1% (wt/vol) α-methyl-D-mannoside to 10^6 to 10^7 CFU/ml. Monolayers were infected with 0.5 ml of bacterial suspension for 1 h at 37°C, washed three times with Hanks balanced salt solution to remove nonadherent bacteria, and lysed by vigorous aspiration with a Pasteur pipette with phosphate-buffered saline containing 0.1% (wt/vol) sodium deoxycholate. Samples were then diluted and plated on L agar plates.

Henle-407 and CHO cell invasion was performed by allowing bacterial adherence as described above, except that monolayers were overlaid with Eagle minimal essential medium containing 50 μg of gentamicin per ml to inhibit growth of extracellular bacteria (39). At various times after the addition of gentamicin, monolayers were washed with Hanks balanced salt solution and lysed with deoxycholate, and intracellular bacteria were enumerated as described above.

Serum resistance. Bacteria were diluted in phosphate-buffered saline to approximately 10^6 CFU/ml. Ten microliters of bacteria (approximately 10^4 CFU) was added to 90 μl of serum and incubated at 37°C for 1 h. Samples were then diluted and plated for enumeration of CFU. *E. coli* K-12 was used as a positive control for killing. Normal human serum was absorbed with whole cells of the *S. typhimurium* strains being tested for serum resistance because antibody-dependent, complement-mediated bacteriolysis was detected, although undiluted serum did not agglutinate *S. typhimurium*. Normal rabbit serum and normal guinea pig serum were not absorbed before use. In some experiments with rabbit and guinea pig sera, sera were buffered with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and preequilibrated in a 5% CO_2 atmosphere to control pH of the sera. In these experiments, bactericidal assays were also incubated in 5% CO_2.

Macrophage studies. For in vitro analysis of interactions between *S. typhimurium* and macrophages, the method of Lissner et al. (39) was used. In vivo macrophage assays were based on a procedure described by Briles et al. (8). Mice were injected i.p. with mixtures containing 10^7 CFU each of wild-type and 100-kb plasmid-cured *S. typhimurium* cells in BSG. After 1 and 4 h, mice were killed by CO_2 asphyxiation, and two peritoneal lavages of 5 ml of ice-cold phosphate-buffered saline were collected. Macrophage-associated bacteria were collected by centrifugation of lavage fluids at 130 × *g* for 10 min at room temperature. Macrophage pellets were suspended in 10 ml of distilled water to lyse cells. The lavage supernatant (macrophage-free) and pellet (macrophage-associated) fractions were diluted and plated. Because different amounts of lavage fluid were recovered from individual mice, the recovery of CFU from each mouse was normalized to the total 10-ml lavage volume. The percentage of macrophage-associated bacteria was calculated as follows: % associated bacteria = [(macrophage-associated CFU)/(macrophage-associated CFU + macrophage-free CFU)] × 100. The percent recovery of bacteria was calculated as follows: % recovery = [(macrophage-associated CFU + macrophage-free CFU)/(CFU in inoculum)] × 100.

An in vivo-in vitro macrophage assay was similar to that described by Briles et al. (8). Mice were injected i.p. with *S. typhimurium*, and peritoneal lavages were done as described above 2 h after injection. Lavage fluids were divided into three portions and centrifuged as described above to separate macrophage-associated and macrophage-free fractions.

Two portions of macrophages were then incubated in vitro in RPMI 1640 (Difco) containing 10 μg of gentamicin per ml to inhibit extracellular bacteria (39). Surviving bacteria were quantitated 1 and 3.5 h after incubation in gentamicin by pelleting macrophages and lysing in deoxycholate.

Statistical methods. For comparison of CFU in tissues of mice infected with either wild-type or cured *S. typhimurium*, geometric means were determined and compared in a one-tailed Student *t* test for wild-type CFU being greater than cured CFU. For analysis of mixed infections, geometric means of ratios of wild-type to plasmid-cured CFU from individual mice were compared in a one-tailed Student *t* test for mean of ratios greater than 1:1. Bacterial CFU in tissue culture and macrophage infections were compared by Student *t* test of mean CFU per well in a two-tailed test.

Analysis of LPS. The method of Hitchcock and Brown (28) was used to resolve lipopolysaccharide (LPS) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 12.5% (wt/vol) polyacrylamide gels (24). LPS was stained by the method of Tsai and Frasch (61).

RESULTS

Presence of 100-kb plasmids in *S. typhimurium* strains. All three strains of *S. typhimurium* studied possessed 100-kb plasmids (Fig. 1, lanes a, d, and g). In addition, SL1344 strain χ3339 possessed two other plasmids of 90 and 8 kb (lane g). We used the following three-part nomenclature system for virulence plasmids of the *S. typhimurium* strains in this study. The serotype is identified as *S. typhimurium* by the designation "St." The strain designation ("LT" for LT2-Z, "SR" for SR-11, and "SL" for SL1344) follows. A numerical designation finally identifies the plasmid as being the wild type (100) or a derivative (101 for a particular Tn*mini-tet* insertion, etc.). The wild-type plasmid of strain SR-11 therefore is pStSR100.

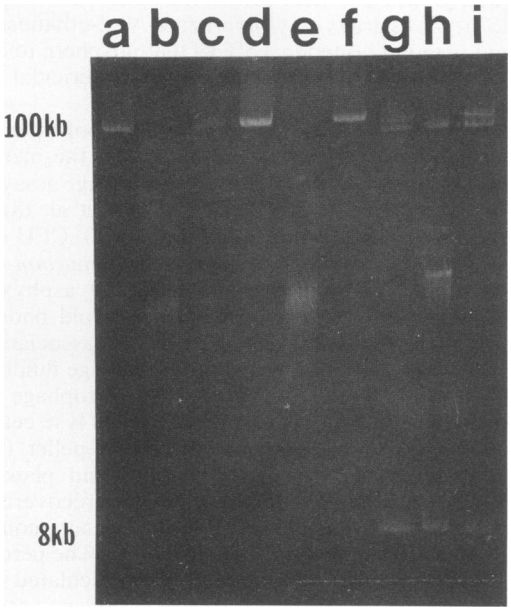

FIG. 1. Plasmid content of *S. typhimurium* strains. Plasmid DNA was extracted by the method of Birnboim (4), purified by cesium chloride density gradient centrifugation, resolved in a 0.5% (wt/vol) agarose gel, and stained with ethidium bromide. χ3344 and χ3337 lysates were extracted with phenol-chloroform-ether and not subjected to density gradient centrifugation. Lanes: a, χ3000 (pStLT100); b, χ3344; c, χ3347(pStLT101); d, χ3306(pStSR100); e, χ3337; f, χ3338(pStSR101); g, χ3339(pStSL100, 90 kb, 8 kb); h, χ3340(90 kb, 8 kb); i, χ3351(pStSR101, 90 kb, 8 kb). Marker plasmids were R1*drd* (100 kb) and dimeric pACYC184 (8 kb) (data not shown).

Cesium chloride gradient-purified plasmid preparations were analyzed by restriction enzyme digestion, and identical profiles were obtained for the 100-kb plasmids from all three strains with *Hin*dIII and *Eco*RI (data not shown). Therefore, the 100-kb plasmids of *S. typhimurium* LT2-Z, SR-11, and SL1344 were very similar, if not identical, in structure.

Strain construction. We used the transposon Tn*mini-tet* described by Way et al. (62) to label the 100-kb *S. typhimurium* plasmid with a Tet^r marker. Tn*mini-tet*, a Tn*10* derivative, does not possess the IS*10R* transposase gene within the inverted repeats, hence the transposase gene is lost upon transposition from the donor plasmid, pNK861, and cannot subsequently mediate transposition or deletion of Tn*mini-tet*. Therefore, Tn*mini-tet* insertions are much more stable than those of the parent, Tn*10*. This fact would essentially preclude selection of deletion events as opposed to plasmid-curing events.

The 100-kb plasmid of strain χ3000, pStLT100, was labeled with Tn*mini-tet*, and the labeled plasmid was designated pStLT101. During growth of χ3000(pStLT101) in novobiocin (200 μg/ml) or at 43°C, derivatives cured of the 100-kb plasmid were detected at a rate of 10^{-6} to 10^{-7} cell^{-1} generation^{-1}. χ3000 cured of the 100-kb plasmid was designated χ3344. Similarly, χ3306(pStSR101) and χ3339(pStSL101) were cured of their Tn*mini-tet*-labeled, 100-kb plasmids resulting in χ3337 and χ3340, respectively. Curing of 100-kb plasmids was confirmed by agarose gel electrophoresis of plasmid DNA from cleared lysates (Fig. 1, lanes b, e, and h) and hybridization of minilysates in Southern blots and lysed bacteria in colony blots with ^{32}P-labeled pStSR100 (data not shown). Lack of hybridization in Southern blots or colony blots indicated that no chromosomally integrated plasmid was present in the cured derivatives.

Tn*mini-tet*-labeled plasmids of strain χ3000, pStLT101, and strain χ3306, pStSR101, were reintroduced into cured derivatives by transformation, yielding strains χ3347 and χ3338, respectively. pStSR101 was transformed into χ3340, yielding χ3351 (Table 1, Fig. 1).

Mouse virulence. The p.o. LD_{50}s in BALB/c mice of each of the wild-type, cured, and retransformed derivatives were determined by the method of Reed and Muench (47). The p.o. LD_{50}s of wild-type χ3306 and χ3339 were much lower than those of the respective cured derivatives (Table 2). Mice infected with χ3337 and χ3340 became sick, and some died. The LD_{50}s of the 100-kb plasmid-retransformed derivatives were similar to those of the parental wild-type strains. This confirmed that the genetic lesion of cured derivatives was, in fact, loss of the 100-kb plasmid. Wild-type LT2-Z was avirulent by the p.o. route.

i.p. LD_{50}s of wild-type and cured derivatives were not as different as were p.o. LD_{50}s (Table 2). The i.p. LD_{50}s of χ3306, χ3339, and χ3337 were <50 CFU, with greater than 90% mortality achieved with this inoculum. However, the mean time to death was greater for χ3337 (12.3 days) relative to χ3306 (7.0 days) ($P < 0.001$, one-tailed Student *t* test). In contrast, the i.p. LD_{50} of 100-kb plasmid-cured SL1344 strain χ3340 was notably raised over that of χ3339. Reintroduction of the Tn*mini-tet*-labeled, 100-kb plasmid pStSR101 into χ3340 restored the wild-type i.p. LD_{50}. The i.p. LD_{50}s for wild-type and cured LT2-Z strains were equal and much higher than those of SR-11 and SL1344.

Effects of plasmid exchange between strains SR-11 and

TABLE 2. Mouse LD_{50}s[a] for wild-type and 100-kb plasmid-cured *S. typhimurium*

Strain	100-kb plasmid	LD_{50} (CFU)	
		p.o.	i.p.
SR-11			
χ3306	+	3×10^5	<50
χ3337	−	$>10^8$	<50[b]
χ3338	+	10^5	<50
SL1344			
χ3339	+	6×10^4	<50
χ3340	−	$>6 \times 10^8$	400
χ3351	+	$<5 \times 10^4$	<50
LT2-Z			
χ3000	+	$>10^8$	2×10^3
χ3344	−	NT[c]	2×10^3
χ3347	+	$>10^9$	NT

[a] Determined for female BALB/c mice by the method of Reed and Muench (47).

[b] The mean time to death for χ3337 (12.3 days) was greater than that of χ3306 (7.0 days) ($P < 0.001$, Student *t* test).

[c] NT, Not tested.

LT2-Z. To determine whether the 100-kb plasmid of strain LT2-Z had a role in the avirulence of this strain, the Tn*mini-tet* derivatives of the 100-kb plasmids of LT2-Z and SR-11 were transformed into the heterologous cured strains. Mice infected p.o. with 10^9 CFU of SR-11 possessing either pStLT101 or pStSR101 died by 7 days postinoculation, whereas LT2-Z was avirulent with either plasmid. Therefore, the avirulence of LT2-Z was not due to defects in its 100-kb plasmid.

Pathogenesis after p.o. inoculation. The more distinct differences in virulence between wild-type and cured derivatives by the p.o. route versus the i.p. route raised the possibilty that the 100-kb plasmid was involved in virulence in the gut instead of during later stages of invasive disease. To examine this possibility, mice were infected p.o. with either wild-type SR-11 strain χ3306 or 100-kb plasmid-cured SR-11 strain χ3337, and Peyer's patches and spleens were examined for *S. typhimurium* at various times postinfection. The composite results of three experiments are presented in Fig. 2. At 1 and 2 days postinoculation, the CFU in Peyer's patches increased to 10^3 to 10^4 CFU, and CFU in spleens were low. At 3 days postinoculation, Peyer's patches and spleens possessed significantly more wild-type χ3306 than cured χ3337. The difference in CFU in Peyer's patches was not consistently observed in different experiments and, as the data for other time points indicate, was temporary. However, CFU in spleens remained significantly different with χ3306 outnumbering χ3337 in increasing amounts until mice infected with χ3306 died by 8 days postinoculation. χ3337 in spleens reached levels on the order of 10^4 CFU and remained detectable in spleens as long as 25 days postinoculation. Thus, the primary difference in pathogenesis after p.o. inoculation between wild-type and virulence plasmid-cured *S. typhimurium* SR-11 was in the numbers of *S. typhimurium* reaching, multiplying, or surviving in spleens.

To more precisely compare the relative virulences of wild-type and plasmid-cured SR-11 strains, mixed-infection experiments with Tet^r, wild-type SR-11 strain χ3456 and Nal^r, plasmid-cured SR-11 strain χ3337 were done. In addition, ratios of CFU were determined for mesenteric lymph nodes, which are intermediate in the invasive process from Peyer's patches to spleens. Composite results of three experiments are presented in Fig. 3. No significant differences in CFU in Peyer's patches or spleens were detectable in mice with mixed infections until 3 days postinoculation, when wild-type/cured ratios in Peyer's patches, mesenteric lymph nodes, and spleens were 11:1, 200:1, and 79:1, respectively. At 4 days postinoculation, only the ratio for spleens was significantly greater than 1.0 (ratio, 160:1), whereas Peyer's patches and mesenteric lymph nodes had ratios of 1.5:1 and 13:1, respectively. Again, as observed in mice with separate infections, CFU in Peyer's patches were approximately equal for wild-type and cured SR-11 strains after the transient differences noted at 3 days postinoculation. At 5 days postinoculation, mesenteric lymph nodes and spleens possessed ratios of 200:1 and 1,600:1, respectively; the Peyer's patch ratio was 1.1:1. At 7 days postinoculation, a single mouse survived with Peyer's patch, mesenteric lymph node, and spleen ratios of 2.1:1, 290:1, and 210:1, respectively. The greatest differences in CFU in the various organs were in spleens and mesenteric lymph nodes relative to Peyer's patches. At 3, 4, and 5 days postinoculation, spleen

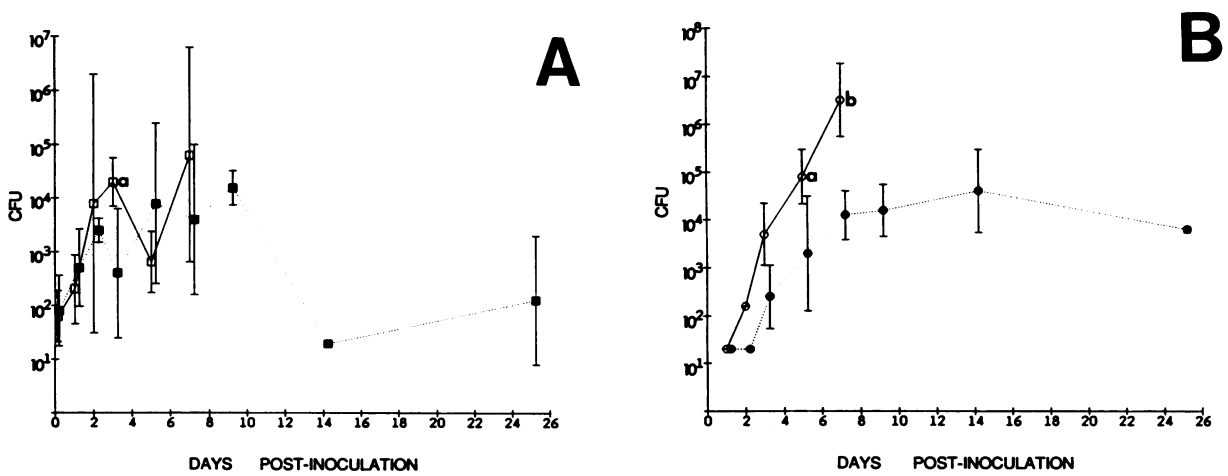

FIG. 2. Total CFU in (A) Peyer's patches and (B) spleens after p.o. inoculation of mice with *S. typhimurium* SR-11 strains χ3306(pStSR100) (□, ○) and χ3337 (plasmid cured) (■, ●). Results are given as geometric means ± standard deviations for two to seven mice. *P* values in the one-tailed Student *t* test for CFU χ3306 greater than χ3337: a, <0.0125; b, <0.0005. All mice infected with χ3306 died by 8 days postinoculation.

ratios were significantly greater than Peyer's patch ratios; at 3 and 5 days postinoculation, mesenteric lymph node ratios were significantly greater than Peyer's patch ratios.

The pathogenesis of wild-type SL1344 strain χ3339 and 100-kb plasmid-cured SL1344 strain χ3340 was investigated in mice infected p.o. Composite results of two experiments are presented in Fig. 4. Results very similar to those obtained with strain SR-11 were obtained. The greatest effect of curing was invasion to mesenteric lymph nodes and spleens and not colonization of Peyer's patches. Furthermore, 100-kb plasmid-cured strain SL1344 survived in Peyer's patches and spleens for extended periods of time after p.o. inoculation.

Infection of spleens after i.v. inoculation. To more precisely examine growth of *S. typhimurium* in spleens, mice were inoculated i.v. with 10^5 CFU of strains χ3456 and χ3337. Both strains were equally cleared to spleens 1 h postinoculation (Table 3); thus, the presence of the 100 kb plasmid did not affect clearance from the blood. Other mice were inoculated i.v. with 1×10^3 to 4×10^3 CFU of mixtures of χ3456 and χ3337. No significant differences in CFU in spleens were detected at 4 days postinoculation (Table 3). However, at 6 to 7 days postinoculation, significantly more wild-type χ3456 than cured χ3337 were present in spleens, and both strains had undergone net multiplication. We did not detect synergy between χ3456 and χ3337 in mixed i.v. infections; at 6 days postinoculation, mice infected with 2×10^3 CFU of χ3337 alone or χ3337 mixed with equal amounts of χ3456 attained mean spleen levels of 2.3×10^5 CFU for χ3337.

Macrophage studies. The great differences in the abilities of wild-type versus 100-kb plasmid-cured *S. typhimurium* to invade to mesenteric lymph nodes and spleens suggested a role for the plasmid in interactions between *S. typhimurium* and macrophages. A variety of experimental parameters were used to examine the fate of *S. typhimurium* after interaction with peritoneal macrophages of BALB/c mice in vitro and in vivo.

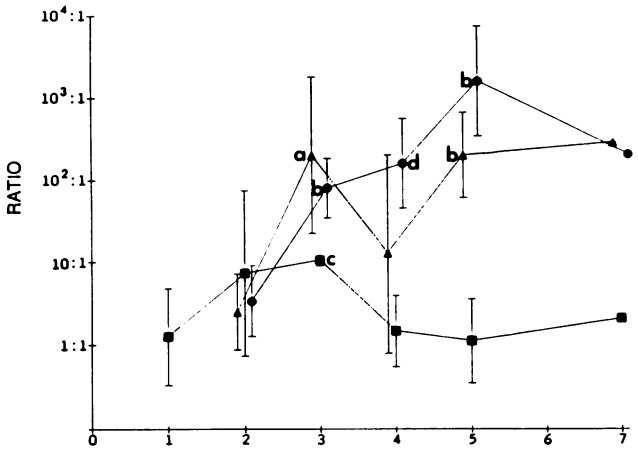

FIG. 3. Mixed p.o. infection of mice with wild-type and 100-kb plasmid-cured SR-11 strains. Results are given as geometric means ± standard deviations of ratios of wild-type χ3456 to cured χ3337 recovered from Peyer's patches (■), mesenteric lymph nodes (▲), and spleens (●) for three to seven mice (one mouse at 7 days postinoculation). *P* values in the one-tailed Student *t* test for geometric mean of ratios greater than 1:1: a, <0.05; b, <0.0125; c, <0.0025; d, <0.0005. The ratio of spleens was greater than ratio of Peyer's patches at 3 days ($P < 0.0125$), 4 days ($P < 0.0005$), and 5 days ($P < 0.0025$) postinfection. The ratio of mesenteric lymph nodes was greater than ratio of Peyer's patches at 3 days ($P < 0.05$) and 5 days ($P < 0.005$) postinfection.

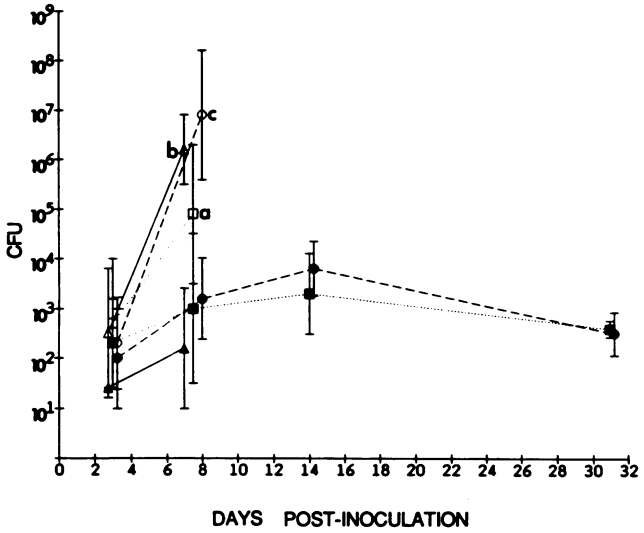

FIG. 4. Total CFU after p.o. inoculation of mice with SL1344 strains χ3339 (□, △, ○) and χ3340 (■, ▲, ●) in Peyer's patches (□, ■), mesenteric lymph nodes (△, ▲), and spleens (○, ●). Results are given as geometric means ± standard deviations for two to seven mice. *P* values in a one-tailed Student *t* test for CFU χ3339 greater than χ3340: a, <0.025 (Peyer's patches); b, <0.0125 (mesenteric lymph nodes); c, <0.0005 (spleens). All mice infected with χ3339 died by 14 days postinoculation.

For in vitro analysis, the method of Lissner et al. (39) was used. Wild-type strain χ3306 and 100-kb plasmid-cured strain χ3337 derived from *S. typhimurium* SR-11 were phagocytosed and survived equally in murine peritoneal macrophages (data not shown). We also examined *S. typhimurium*-macrophage interactions in vitro with proteose peptone- and thioglycholate-elicited macrophages and macrophage cell lines $P388D_1$ (34), IC21 (40), and J774 (17, 46) and did not detect differences in phagocytosis or survival between wild-type and plasmid-cured *S. typhimurium*.

To alleviate possible artifacts involved in infection of peritoneal macrophages in vitro, we examined phagocytosis and killing of *S. typhimurium* in vivo by murine peritoneal macrophages from mice inoculated i.p. by procedures similar to those of Briles et al. (8). At 1 h postinoculation of mice i.p. with 10^7 CFU of χ3456 and χ3337, lavage fluids contained greater than 100% of the inocula of each strain (Table 4). More χ3456 than χ3337 organisms were recovered 1 h postinfection, primarily in the macrophage-free CFU. However, the percent CFU macrophage-associated was not different between χ3456 and χ3337, indicating equivalent

TABLE 3. Infection of spleens after i.v. inoculation of mice[a]

Strain	Mean CFU/spleen at time postinoculation:		
	1 h[b]	4 days[c]	6 to 7 days[c]
χ3456	1.9×10^5	4.6×10^4	2.7×10^6
χ3337	1.4×10^5	2.5×10^4	2.3×10^5

[a] Mice were inoculated i.v. with equal mixtures of wild-type χ3456 and 100-kb plasmid-cured χ3337; three to five mice were used per group.
[b] Inoculum was 5×10^5 CFU each of χ3456 and χ3337.
[c] Inoculum was 1×10^4 to 4×10^4 CFU each of χ3456 and χ3337 (equal inocula were given with each experiment). $P < 0.0025$ at 6 to 7 days in one-tailed Student *t* test for ratios of >1:1.

TABLE 4. In vivo peritoneal macrophage (Mϕ) infection with SR-11 strains[a]

Strain	Time (h) postinfection	Mϕ-free CFU	Mϕ-associated CFU	% CFU Mϕ associated	% Recovery
χ3456	1	$3.0 \times 10^7 \pm 7.0 \times 10^6$	$1.2 \times 10^7 \pm 3.4 \times 10^6$	29 ± 4.3	187 ± 41
χ3337	1	$1.8 \times 10^7 \pm 6.3 \times 10^6$	$9.0 \times 10^6 \pm 2.1 \times 10^6$	32 ± 6.9	115 ± 34
χ3456	4	$9.9 \times 10^7 \pm 5.0 \times 10^7$	$2.8 \times 10^7 \pm 1.9 \times 10^7$	22 ± 8	552 ± 274
χ3337	4	$5.3 \times 10^7 \pm 3.7 \times 10^7$	$1.9 \times 10^7 \pm 1.5 \times 10^7$	27 ± 12	314 ± 218

[a] Mice were infected i.p. with 10^7 CFU each of wild-type SR-11 strain χ3456 and 100-kb plasmid-cured SR-11 strain χ3337. At 1 and 4 h later, peritoneal cavities were lavaged with phosphate-buffered saline, and lavage fluids were centrifuged to obtained macrophage-free (supernatant) and macrophage-associated (pellet) fractions. Results are given as means ± standard deviations, with six mice per group at 1 h postinfection and five mice per group 4 h postinfection. At 1 h postinfection, P values in the two-tailed Student t test were <0.05 for macrophage-free CFU and percent recovery.

phagocytosis of the two strains. At 4 h postinfection, no differences were observed between χ3456 and χ3337 in either percent recovery, macrophage-associated CFU, macrophage-free CFU, or the percent macrophage-associated CFU.

We also examined salmonella-macrophage interactions in an in vivo-in vitro assay in which phagocytosis occurs in vivo and survival of phagocytosed bacteria is followed in vitro (8). As above, both wild-type and cured SR-11 strains were phagocytosed equally in vivo, and both strains survived (i.e., multiplied) intracellularly in macrophages in vitro (data not shown). Thus, all three assay systems—in vitro, in vivo, and in vivo-in vitro—demonstrated no differences in phagocytosis and survival in macrophages between wild-type and virulence plasmid-cured *S. typhimurium*.

Serum resistance. *S. typhimurium* is resistant to complement-mediated bacteriolysis of serum (37, 38). We examined the role of the 100-kb plasmid in serum resistance with normal human serum, rabbit serum, and guinea pig serum. In all experiments, the positive control for complement activity was inclusion of serum-sensitive *E. coli* K-12, and the negative control was lack of killing of K-12 by heat-inactivated serum. To prevent a rise in pH caused by CO_2 evolving from sera exposed to the ambient atmosphere, some sera were buffered with 20 mM HEPES and pre-equilibrated in a 5% CO_2 atmosphere, and assays were incubated in 5% CO_2 (61a). This procedure maintained a serum pH of 7.0 to 7.5, as opposed to a pH of 8.5 to 9.0, in unbuffered sera exposed to the ambient atmosphere. It should be noted that equivalent results were obtained with assays conducted in normal atmosphere and under pH-controlled, CO_2 conditions. With all three sera tested and with all three pairs of wild-type and plasmid-cured *S. typhimurium*, similar resistances to normal sera were exhibited (Table 5). *S. typhimurium* SR-11 and SL1344 experienced net killing in absorbed human serum, whereas strain LT2-Z experienced net growth. These decreases in CFU of 17 to 33% in human serum probably reflected inefficient adsorption of sera and were minor relative to the *E. coli* K-12 control, which had a decrease of greater than 99.9%. When wild-type and cured *S. typhimurium* SR-11 strains were incubated in heat-inactivated normal human serum, net growth occurred (data not shown) as was found with *E. coli* K-12 (Table 5). With normal rabbit and guinea pig sera, all *S. typhimurium* strains experienced net growth. The growth rates of wild-type and 100-kb plasmid-cured SR-11 strains in normal and heat-inactivated rabbit serum were equal for 5 h (data not shown). These experiments demonstrated that the 100-kb plasmid was not involved in serum resistance of *S. typhimurium*, in contrast to results of others (22, 27, 61a).

Infection of Henle-407, HEp-2 and CHO cells. Jones et al. (33) reported that the cryptic (virulence) plasmid of *S. typhimurium* was involved in mannose-resistant adherence of *S. typhimurium* to HeLa cells. We examined the role of 100-kb plasmids of our three pairs of *S. typhimurium* strains in mannose-resistant adherence to two human-derived cell lines, Henle-407 (intestinal epithelial carcinoma) and HEp-2 (laryngeal carcinoma). No differences in mannose-resistant adherence were detected between wild-type and plasmid-cured *S. typhimurium* for either Henle-407 cells or HEp-2 cells (data not shown). Wild-type and plasmid-cured SR-11 strains invaded Henle-407 cells equally (data not shown).

We found that although *S. typhimurium* invaded Henle-407 cells no gross intracellular multiplication occurred over 24 to 48 h postinfection. A similar result was obtained by Small et al. (56) for *S. typhimurium* and HEp-2 cells. We therefore tested the ability of wild-type and 100-kb plasmid-cured *S. typhimurium* to multiply intracellularly in CHO cells. Wild-type and plasmid-cured SR-11 strains adhered to and invaded CHO cells equally (Table 6). In addition, intracellular growth rates from 2 or 3 h postinfection to 20 to 22 h postinfection were equal between wild-type and plasmid-cured SR-11 strains. Thus, the 100-kb plasmid did not affect the ability of *S. typhimurium* to multiply within CHO cells.

TABLE 5. Serum resistance of wild-type and 100-kb plasmid-cured *S. typhimurium*

Strain	100-kb plasmid	% CFU (mean ± SD, n = 2) remaining 1 h after incubation in serum[a]:		
		Human	Rabbit	Guinea pig
S. typhimurium SR-11				
χ3306	+	83.2 ± 0.5	287 ± 3	119 ± 19
χ3337	−	74 ± 15	132 ± 7	103 ± 11
SL1344				
χ3339	+	67.2 ± 6.6	800 ± 120	570 ± 110
χ3340	−	73 ± 21	610 ± 40	510 ± 65
LT2-Z				
χ3000	+	120 ± 20	560 ± 76	410 ± 40
χ3344	−	186 ± 13	220 ± 40	290 ± 40
E. coli K-12				
χ2934		<0.1	<0.1	0.3
χ2934 (Δ serum)[b]		340 ± 20	580 ± 80	420 ± 115

[a] Human serum was absorbed with the homologous wild-type *S. typhimurium* strain. Rabbit and guinea pig sera were buffered with 20 mM HEPES and incubated in a 5% CO_2 atmosphere for assays.

[b] Heat-inactivated serum.

TABLE 6. Infection of CHO cells

Strain	100-kb plasmid	% Adherence[a]	% Invasion[b]	% Growth[c]	Generation time (h)
χ3306	+	3.1 ± 0.5	122 ± 19	2,500 ± 600	3.8
χ3337	−	2.9 ± 0.5	150 ± 20	2,700 ± 1,200	3.7

[a] Percentage of inoculum adherent to cells 1 h after incubation in the presence of 1% α-methyl-D-mannoside (mean ± standard deviation; $n = 7$).
[b] Percentage of CFU recovered from CHO cells 2 or 3 h after incubation in 50 μg of gentamicin per ml relative to adherent CFU after 1 h in the presence of 1% α-methyl-D-mannoside (mean ± standard deviation; $n = 7$).
[c] Percentage of CFU recovered from CHO cells after incubation in gentamicin for 20 to 22 h relative to CFU at 2 or 3 h (mean ± standard deviation; $n = 7$).

LPS. Hackett et al. (23) reported that their 100-kb plasmid-cured derivative of strain LT2 possessed incomplete, rough LPS, as detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and serotype analysis. We examined the LPS structure of all three pairs of wild-type and cured *S. typhimurium* and found that all strains possessed complete, smooth LPS with no observable differences (Fig. 5). *E. coli* K-12 and *S. typhimurium* χ3477 were included as controls for incomplete LPS.

Other phenotypes examined in relation to the 100-kb plasmid. Some pathogens possess plasmids that confer phenotypes such as binding of Congo red dye (18, 43, 49), the ability to sequester iron (13, 64), and the ability to utilize citrate (50, 58). We found that the presence of 100-kb plasmids was not associated with the ability of *S. typhimurium* to bind Congo red dye when present in brain heart infusion agar; wild-type and plasmid-cured strains bound small amounts of the dye. Both wild-type and cured *S. typhimurium* grew equally well in the presence or absence of 200 mM α,α′-dipyridyl in minimal salts-glucose medium (13), demonstrating no relation of the plasmid to acquisition of iron. Wild-type and plasmid-cured *S. typhimurium* were citrate positive when tested on Simmons citrate medium (55), demonstrating lack of involvement of the 100-kb plasmid in this characteristic. Because *S. typhimurium* probably encounters an acidic environment within phagolysosomes of infected macrophages and epithelial cells, we investigated the abilities of wild-type and cured *S. typhimurium* to grow at low pH in minimal and complex media. No differences in growth rates between wild-type and plasmid-cured SR-11 strains were observed in L broth or minimal salts-glucose medium (14) at pH 5.0 and 7.0; neither strain survived at pH 3.0.

DISCUSSION

We have examined the role of the 100-kb plasmid of *S. typhimurium* in mouse virulence. Our results confirm those of some investigators and conflict with the results of others. We confirmed the results of Hackett et al. (22) and Pardon et al. (45) that the presence of the 100-kb plasmid is not necessary for *S. typhimurium* to efficiently colonize the Peyer's patches after p.o. inoculation of BALB/c mice. We found that wild-type *S. typhimurium* infected mesenteric lymph nodes and spleens more efficiently than did cured derivatives. In partial agreement, Pardon et al. (45) found that wild-type strains infected spleens more efficiently than did cured derivatives, although wild-type and plasmid-cured *S. typhimurium* infected mesenteric lymph nodes equally after p.o. inoculation. We also found that cured derivatives reached spleen levels as high as 10^4 CFU and remained detectable in spleens for as long as 31 days postinoculation (Fig. 2 and 4). In a comprehensive study of pathogenesis of wild-type and virulence plasmid-cured *Salmonella dublin*, Heffernan et al. (26) recently obtained results very similar to ours reported here with *S. typhimurium*. We have used the invasiveness to spleens conferred by the 100-kb plasmid after p.o. inoculation to select virulence-conferring recombinant clones of the 100-kb plasmid in cured *S. typhimurium* (P. A. Gulig and R. Curtiss III, submitted for publication).

Virulence plasmid-cured *S. typhimurium* retained significant virulence by the i.p. route of inoculation (Table 2). In contrast, the Tn*10* insertion mutant of Fields et al. (21) was significantly attenuated by the i.p. route (LD_{50}, $>10^3$ CFU). However, their parental strain and insertion mutant possessed rough LPS, which could have acted in concert with the Tn*10* insertion to synergistically decrease the virulence of the plasmid mutant. It is also important to note that none of our cured strains hybridized with ^{32}P-labeled virulence plasmid, so the presence of chromosomally integrated copies of the plasmid (3, 33), which could contribute to virulence, was ruled out. Furthermore, isolates of cured derivatives from diseased or dead mice still lacked the 100-kb plasmid. We hypothesize that 100-kb plasmid-cured *S. typhimurium* retains virulence by the i.p. route because the bacteria rapidly multiply extracellularly and are not efficiently phagocytosed in the peritoneal cavity (Table 4); hence an overwhelming infection develops before mice can check the infection by clearing bacteria to macrophages. When administered by the p.o. route, all or most of the invading bacteria are presumed to be intracellular upon reaching the mesenteric lymph nodes.

Animal infection studies demonstrating reduced invasiveness of cured strains to mesenteric lymph nodes and spleens of mice p.o. infected suggested that the 100-kb plasmid might be involved in the ability of *S. typhimurium* to resist phagocytosis or killing by murine macrophages or might be involved in the ability to multiply within macrophages. However, wild-type and cured *S. typhimurium* were

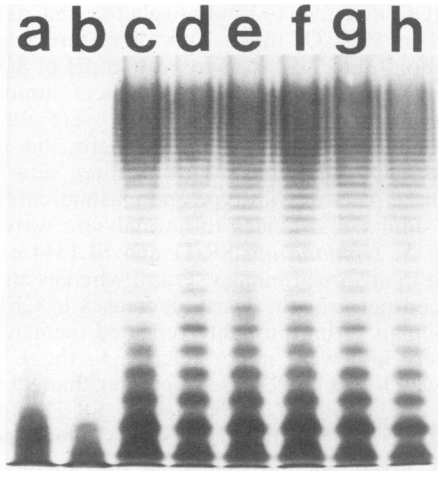

FIG. 5. LPS of *S. typhimurium* strains. Bacterial cells were boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (24), diluted into sample buffer without sodium dodecyl sulfate (final concentration of sodium dodecyl sulfate, 0.2% [wt/vol]), and digested with proteinase K for 1 h at room temperature. Samples were resolved in a 12.5% polyacrylamide gel and stained by the method of Tsai and Frasch (61). Lanes: a, *E. coli* K-12, Ra chemotype; b, χ3477, Rc chemotype; c, χ3000 (100-kb plasmid); d, χ3344; e, χ3306 (100-kb plasmid); f, χ3337; g, χ3339 (100-kb plasmid); and h, χ3340.

phagocytosed and killed to equal degrees up to 5 h postinfection in in vivo and in vitro assays. Because of the short infection times, these experiments could not address possible differences in growth rates of wild-type and cured *S. typhimurium* within infected macrophages. Such analysis awaits further investigation. The results of Fields et al. (21) that a Tn*10* insertion into the 100-kb plasmid of *S. typhimurium* rendered their strain more susceptible to thioglycholate-elicited macrophages in vitro were not inconsistent with the possibility that the plasmid is involved with multiplication within macrophages, rather than resistance to killing by macrophages. Another possibility is that the virulence plasmid mediates some form of immunosuppression, as has been documented for *S. typhimurium* (20, 35).

We have confirmed that the *S. typhimurium* virulence plasmid is not involved in mannose-resistant adherence to tissue culture cells (22) (Table 6). We extended this observation to demonstrate that both wild-type and cured *S. typhimurium* attach to, invade, and replicate in CHO cells equally (Table 6). Therefore, the *S. typhimurium* virulence plasmid does not appear to affect interactions of *S. typhimurium* with nonphagocytic cells represented by these cell lines. In experiments not shown, we detected equal numbers of wild-type and cured *S. typhimurium* cells in intestinal wall segments devoid of Peyer's patches after p.o. inoculation of mice. This result was expected from the lack of involvement of the virulence plasmid in adherence to, invasion of, and multiplication in tissue culture cells in vitro.

The greatest subject of controversy in studies of the *S. typhimurium* virulence plasmid is its role in serum resistance. Hackett et al. (22) reported that their cured LT2 strain was extremely sensitive to normal human serum and was quickly cleared from peritoneal cavities of mice after i.p. inoculation. Our data conflict with theirs; we found no such differences in the sensitivities of three different *S. typhimurium* strains to three types of serum (Table 5), and wild-type and cured *S. typhimurium* survived equally in peritoneal cavities after i.p. inoculation (Table 4). In addition, the i.p. LD_{50}s of wild-type and cured SR-11 and SL1344 were very low, which would be unexpected if these strains were serum sensitive or rapidly cleared from peritoneal cavities. Our differences with Hackett et al. concerning serum resistance may be due to the facts that their cured strain, as opposed to its parent, possessed primarily incomplete, rough LPS (23) and their plasmid-containing parental strain was relatively avirulent (i.p. LD_{50}, approximately 5×10^5 CFU [22]). All wild-type and 100-kb plasmid-cured strains in our study possessed complete LPS (Fig. 5), and complete LPS of *S. typhimurium* has been shown to be necessary for resistance to complement-mediated bacteriolysis by normal serum (48). Most importantly, Hackett and co-workers did not demonstrate that reintroduction of the plasmid into their cured derivative restored wild-type virulence properties. In the absence of such a control, the exact genetic lesion responsible for the avirulence of a cured derivative cannot be known. In this regard, we have produced 100-kb plasmid-cured derivatives that did not regain virulence after reintroduction of the plasmid (data not shown). It is interesting to note, however, that Hackett et al. recently reported cloning a gene from the 100-kb plasmid that conferred serum resistance to both plasmid-cured *S. typhimurium* and *E. coli* K-12 (23). Additionally, Vandenbosch and Jones (61a) reported cloning serum resistance genes from the virulence plasmid. We can only speculate as to our differences with Vandenbosch and Jones concerning serum resistance, especially after we modified our serum resistance assays to control pH in accordance with their methods. We have cloned regions from the 100-kb plasmid which confer wild-type levels of virulence to plasmid-cured *S. typhimurium* (Gulig and Curtiss, submitted), and *E. coli* K-12 strains possessing these virulence-conferring recombinant plasmids did not acquire serum resistance.

A possible explanation for differences noted in the virulence of 100-kb plasmid-cured strains of different laboratories may be that the plasmid duplicates virulence functions encoded primarily on the chromosome. Hence, strains with defects in particular chromosomal genes will depend more heavily on the plasmid, and loss of the plasmid will have a greater effect on virulence. For example, the rough LPS resulting in serum sensitivity of the cured LT2 strain of Hackett et al. (22, 23) may allow detection of possible serum resistance-enhancing genes encoded on the plasmid.

Our laboratory is involved in the construction of live, attenuated *S. typhimurium* vaccine strains to stimulate mucosal immunity (16, 16a). It is believed that priming the immune system for mucosal immunity occurs at the Peyer's patches (14, 32). Therefore, efficient infection of Peyer's patches would be an important characteristic of an attenuated, live vaccine strain. Since curing *S. typhimurium* of the 100-kb virulence plasmid inhibits invasive infection to mesenteric lymph nodes and spleens without eliminating infection of Peyer's patches, curing *S. typhimurium* of the virulence plasmid appears to be a desirable genetic attribute for a vaccine strain. However, the residual invasive potential of cured derivatives after p.o. inoculation and the significant virulence of cured derivatives after parenteral inoculation necessitate additional genetic mutations to sufficiently attenuate *S. typhimurium* for vaccine development. Nakamura et al. (44) recently examined the use of virulence plasmid-cured derivatives of *S. enteritidis* as vaccines.

The exact role of the 100-kb plasmid in virulence of *S. typhimurium* has not been conclusively determined. It is clear that the plasmid is involved primarily in invasion past the gut after p.o. inoculation of mice; however, cured strains retain significant virulence when administered parenterally. Ongoing studies center on determining how the 100-kb plasmid mediates invasion from the Peyer's patches to mesenteric lymph nodes and spleens, possibly by affecting growth rates in macrophages or mediating immunomodulation.

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